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<p>(54) Title: GENOTYPING CYTOCHROME EXPRESSION</p> <p>(57) Abstract</p> <p>There is disclosed a method of identifying subjects having a high or low drug metabolising phenotype associated with cytochrome CYP3A5 expression, which method comprises screening genomic DNA from said subject for the presence or absence of one or more polymorphic variants in a transcription regulatory region of the sequence encoding CYP3A5. Oligonucleotide molecules for carrying out the screening are also provided.</p>		

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GENOTYPING CYTOCHROME EXPRESSION

The present invention is concerned with an assay and, in particular, with an assay for genotyping a polymorphism predictive of a phenotype associated with cytochrome expression, in this case CYP3A5.

The cytochrome P450 subfamily CYP3A represents one of the most important families of the P450 superfamily and plays a major role in the metabolism of an ever expanding list of therapeutic compounds (23, 24). This family comprises the most abundantly expressed P450s in human livers, and is responsible for the metabolism of over 50% of all clinically used drugs, including the dihydropyridines, cyclosporin, erythromycin and barbiturates (1). Wide inter-individual variation in the metabolism of CYP3A substrates has been noted and is a factor in determining individual drug efficacy. Evidence also exists for the metabolism of an array of lipophilic environmental pollutants, including the activation of pro-carcinogens such as aflatoxin B1 by members of this subfamily (2).

Presently, four CYP3A cDNAs have been identified in humans, CYP3A3, CYP3A4, CYP3A5 and CYP3A7. It is believed that CYP3A3 represents an allelic variant of CYP3A4, whilst CYP3A4 and CYP3A7 are found only in human adult and fetal livers respectively (3). Initial experiments suggested that a polymorphism existed in CYP3A4 (4). However other studies, whilst confirming a wide range of inter-individual variation in CYP3A4 expression have failed to confirm the original bimodality (5, 6). Overlapping substrate specificities between CYP3A4 and CYP3A5 have previously made it difficult to separate metabolism by these isoforms;

consequently little phenotyping data have been produced to study variation in CYP3A5 activity in humans. However, there is evidence for the polymorphic expression of CYP3A5. Use of both immunoblotting and Northern analysis have detected CYP3A5 expression in only 10-30% of human livers (7, 8, 9). More recently, analysis of 30 human liver samples using immunoblotting found that only 3% showed no detectable CYP3A5, whilst a large number had trace amounts, suggesting that a polymorphism in this enzyme may be regulatory as opposed to structural (10). Comparisons of the 5' flanking regions from the CYP3A4, 3A5 and 3A7 genes have identified putative binding sites for several transcriptional regulatory factors common to all isoforms (11, 12, 13). However, the molecular basis, if any, for this inter-individual variation in expression of the CYP3A sub-family members has so far remained unclear. Indeed it has been suggested that the host cellular environment may be a greater determinant of inducibility than gene structure (14). However, the determination of a major genetic component to variant expression and activity, linked to an easy screening method, would be extremely beneficial, not only in providing a predictor of individual response to drugs which are metabolised by these isoforms, but also in facilitating association studies between CYP3A and disease processes.

The delineation of CYP3A4 and CYP3A5 metabolism has been shown to be possible using the sedative midazolam as a probe drug (15). In this case two metabolites are formed, 1-hydroxy midazolam (1-OHM) and 4-hydroxy midazolam (4-OHM). Those samples containing a higher proportion of CYP3A5 compared to CYP3A4 have their

metabolism driven towards the 1-OHM route and therefore show a higher ratio of 1-OHM/4-OHM than those containing only CYP3A4. The present inventors have now established that two polymorphisms, located
5 in putative transcriptional regulatory regions, which caused increased CYP3A5 gene expression and metabolic activity are linked and have developed assays for their detection. These assays will allow prediction of inter-individual variability in response to drugs
10 metabolised by this isoform, as well as facilitating disease association studies.

Therefore, according to a first aspect of the present invention there is provided a method of identifying
15 subjects having a high or low drug metabolising phenotype associated with cytochrome CYP3A5 expression, which method comprises screening for the presence or absence in the genome of a subject a polymorphic variant in a transcription regulatory
20 region, such as, a promoter or enhancer adjacent the region encoding CYP3A5. Preferably, the method involves screening for a variant in a recognition site for a transcription factor of said regulatory region, and even more preferably in an activator protein-3
25 motif or a basic transcription element. Even more preferably, the method involves screening for a variant at any one of positions -475 or -147 of the DNA of the 5' flanking region adjacent to the region encoding CYP3A5 the sequence of which flanking region
30 is illustrated in Figure 7 and preferably, for both the variants at positions -475 and -147.

In one embodiment of the method of the invention genomic DNA is amplified, preferably by the polymerase

chain reaction using oligonucleotide molecules capable of hybridising selectively to the wild type sequence or the variant sequences, such that generation of amplified DNA from said molecules will indicate whether said wild type or mutation is present. In this method PCR primers hybridise either to the mutated or wild type sequence, but not both. Amplification of the DNA of the respective mutation or wild type genotype using the respective primers will provide an indication of the presence of the wild type or mutated nucleotide mutations.

A further method of the invention advantageously utilises oligonucleotide molecules as primers which, in addition to hybridising to the site of interest, are capable of introducing a restriction site which is absent in either the wild type sequence or polymorphic variants. Therefore, according to a further aspect of the invention, there is provided a method of identifying subjects having a high or low drug metabolising phenotype associated with CYP 3A5 expression, which method comprises 1) amplifying genomic DNA from a subject using oligonucleotide molecules capable of hybridising to the wild type sequence and/or to the polymorphic variant sequence at a location being analysed, which molecules are such that they can introduce a restriction site at said location which is not present in the wild type or variant sequences, and 2) subjecting amplified DNA from step 1 to a restriction enzyme which cleaves the DNA at said restriction site to provide a restriction digest indicative of the presence or absence of said variant.

The method preferably comprises amplifying DNA in a

recognition site for a transcription factor of said regulatory region and preferably in an activator protein-3 motif (AP-3) and/or basic transcription element (BTE). Preferably, the method comprises
5 amplifying DNA spanning any of position -475 or -147, of the regulatory region of CYP 3A5, the sequence of which is illustrated in Figure 7.

The polymorphisms at the positions identified in each
10 of the methods according to the invention comprise T₋₄₇₅ - G and A₋₁₄₇ - G. As presented in the Examples below, the molecule used to detect the variation at A₋₁₄₇ - G is capable of introducing a restriction site for the enzyme *Tai* I only when the wild type A
15 nucleotide is present at position -147. Alternatively, the molecule used to detect the T₋₄₇₅ - G nucleotide variation is capable of introducing a restriction site for the enzyme *Alu* I only when the wild type T nucleotide is present at
20 position -475.

In this embodiment an example of suitable primers is any of 3A5F1 GGGTCTGTCTGGCTGCGC
and 3A5F2 (GGGGTCTGTCTGGCTGAGC)
25 and 3A5R1 (TTTATGTGCTGGAGAAGGACG).

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Using oligonucleotide mismatch primer 3A5R1 creates a *Tai* I recognition site only when the wild type A nucleotide is present at position -147. Digestion of
30 the 369bp product with *Tai* I yields fragments of 349 and 20bp for the wild type sequence, whilst the product remains undigested if a mutant, such as the G nucleotide, is present (Figure 2). Similarly, for the detection of the T₋₄₇₅G mutation a second
35 oligonucleotide mismatch primer 3A5F2 may be used.

This primer introduces a recognition site for the restriction enzyme Alu I when the wild type T is present at position -475, digesting the product to yield fragments of 318, 33 and 18bp. This site is
5 lost when the mutant G nucleotide is present, yielding digestion products of 336 and 33bp (Figure 3).

Known techniques for the scoring of single nucleotide polymorphisms (see review by Schafer, A. J. and
10 Hawkins, J. R. in Nature Biotechnology, Vol 16, pp33-39 (1998) include mass spectrometry, particularly matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS, see Roskey, M. T. et.al., 1996, PNAS USA, 93: 4724-4729), single
15 nucleotide primer extension (Shumaker, J. M. et.al., 1996, Hum. Mutat., 7: 346-354; Pastinen, T. et.al., 1997, Genome Res., 7: 606-614) and DNA chips or microarrays (Underhill, P. A. et.al., 1996, PNAS USA, 93: 196-200; Gilles, P. N. et.al. Nat. Biotech., 1999,
20 17: 365-370). The use of DNA chips or microarrays could enable simultaneous genotyping at many different polymorphic loci in a single individual or the simultaneous genotyping of a single polymorphic locus in multiple individuals.

25 In addition to the above, SNPs are commonly scored using PCR-SSCP based techniques, such as PCR-SSP using allele-specific primers (described by Bunce, 1995). If the SNP results in the abolition or
creation of a restriction site then genotyping can be
30 carried out by performing PCR using non-allele specific primers spanning the polymorphic site and digesting the resultant PCR product using the appropriate restriction enzyme. The known techniques for scoring polymorphisms are of general applicability
35 and it would therefore be readily apparent to persons

skilled in the art that the known techniques could be adapted for the scoring of single nucleotide polymorphisms in the the regulatory region of CYP 3A5.

5 As would be readily apparent to those skilled in the art, genotyping is generally carried out on genomic DNA prepared from a suitable tissue sample obtained from the subject under test. Most preferably, genomic
10 DNA is prepared from a blood sample, according to standard procedures which are well known in the art

Also provided by the present invention is an oligonucleotide of at least 10 contiguous nucleotides to detect polymorphic variants in a 5' regulatory
15 region adjacent the sequence encoding cytochrome CYP3A5 associated with a high or low drug metabolising phenotype. The oligonucleotide is capable of hybridising to a region incorporating either a mutated or wild type nucleotide at position -475 or -147 of
20 said flanking region, such that amplification of said positions will or will not proceed from said primer according to whether or not a polymorphic variant occurs at any of said positions.

25 The oligonucleotide molecules of the invention are preferably from 10 to 50 nucleotides in length, even more preferably from 20-30 nucleotides in length, and may be DNA, RNA or a synthetic nucleic acid, and may be chemically or biochemically modified or may
30 contain non-natural or derivatized nucleotide bases, as will be readily appreciated by those skilled in the art. Possible modifications include, for example, the addition of isotopic or non-isotopic labels, substitution of one or more of the naturally occurring
35 nucleotide bases with an analog, internucleotide

modifications such as uncharged linkages (e.g. methyl phosphonates, phosphoramidates, carbamates, etc.) or charged linkages (e.g. phosphorothioates, phosphorodithioates, etc.). Also included are

5 synthetic molecules that mimic polynucleotides in their ability to bind to a designated sequence to form a stable hybrid. Such molecules are known in the art and include, for example, so-called peptide nucleic acids (PNAs) in which peptide linkages substitute for

10 phosphate linkages in the backbone of the molecule. An oligonucleotide molecule according to the invention may be produced according to techniques well known in the art, such as by chemical synthesis or recombinant means.

15 The oligonucleotide molecules of the invention may be double stranded or single stranded but are preferably single stranded, in which case they may correspond to the sense strand or the antisense strand of the 5' regulatory region of CYP3A5. The

20 oligonucleotides may advantageously be used as probes or as primers to initiate DNA synthesis/DNA amplification. They may also be used in diagnostic kits or the like for detecting the presence of one or more variants alleles of the regulatory region of

25 CYP3A5. These tests generally comprise contacting the probe with a sample of test nucleic acid (usually genomic DNA) under hybridising conditions and detecting for the presence of any duplex or triplex formation between the probe and complementary nucleic

30 acid in the sample. The probes may be anchored to a solid support to facilitate their use in the detection of these variants. Preferably, they are present on an array so that multiple probes can simultaneously hybridize to a single sample of target nucleic acid.

35 The probes can be spotted onto the array or

synthesised *in situ* on the array. (See Lockhart et al., Nature Biotechnology, vol. 14, December 1996 "Expression monitoring by hybridisation to high density oligonucleotide arrays". A single array can
5 contain more than 100, 500 or even 1,000 different probes in discrete locations. Preferably, the oligonucleotides comprise any of the primers 3A5F1, 3A5F2 and 3A5R1 as defined herein.

10 Also provided is a kit to perform the method according to the invention. Preferably, the kit will comprise an oligonucleotide as described herein and even more preferably the kit will further comprise one or more
15 restriction enzymes capable of distinguishing between wild-type or polymorphic variants as defined herein. Preferably, the restriction enzyme comprises *Tai* I or *Alu* I.

According to a further aspect of the invention there
20 is also provided a method of identifying toxic or mutagenic effects of a test compound, such as, a drug, toxin or procarcinogen metabolised by CYP3A5 the method comprising contacting each of a cell having a high drug metabolising phenotype and a cell having a
25 low metabolising phenotype associated with cytochrome CYP3A5 expression, with said test compound and identifying the effects of said compound on each of said high or low drug metabolising phenotype cells or other cells sensitive to said compound. An even
30 further aspect comprises a method of diagnosing susceptibility of an individual to a disease associated with environmental toxins or procarcinogens metabolised by CYP3A5, the method comprising the steps of 1) providing a sample containing DNA, and 2)
35 identifying the presence or absence of a mutation in a

transcription regulatory region adjacent to the DNA sequence encoding CYP3A5 using a reagent capable of distinguishing the presence or absence of a nucleotide in said regulatory site. According to this aspect of the invention, the mutation occurs in a recognition site for a transcription factor of said regulatory region and preferably in an activator protein-3 motif (AP-3) and/or a basic transcription element (BTE). Preferably, the mutation occurs at any of positions - 475 and -147 of the regulatory region and even more preferably at both positions where the mutation may be T₋₄₇₅G or A₋₁₄₇G.

Advantageously, it is also envisaged that the regulatory region of the 5' flanking region can be used to identify or purify transcription factors which bind to the 5' region including the respective polymorphic variants. Thus, according to a further aspect of the invention, there is provided a method of identifying transcription factors capable of binding to a DNA fragment from a transcription regulatory region adjacent DNA encoding cytochrome CYP3A5, said method comprising contacting said DNA fragment including said transcription regulatory region with potential transcription factors and identifying any transcription factor complexed to said DNA fragments.

Using the transcription regulatory fragment it is possible to identify compounds or agents which exhibit or exert their effect on the transcription regulatory region of CYP3A5. Thus, there is provided according to this aspect of the invention a method of identifying compounds acting on a transcription regulatory region adjacent to a DNA sequence encoding CYP3A5, the method comprising transforming a cell with

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a DNA construct comprising the sequence of said regulatory region, and which regulatory region is operably linked to a sequence encoding a reporter molecule, contacting said cell with a test compound and identifying any expression of said reporter molecule. Preferably, said cell is expressing CYP3A5 or is showing CYP3A5 activity.

Also provided by the invention is a method of purification of transcription factors from a sample which are capable of binding to DNA from a transcription regulatory region adjacent a DNA sequence encoding cytochrome CYP3A5, the method comprising contacting a DNA fragment including said transcriptional regulatory region with a mixture of transcription factors and identifying any complexes of said transcription factors and said fragment.

An even further aspect of the invention comprises a method of providing a measure of activity of a transcription regulatory region adjacent to DNA encoding cytochrome CYP3A5 or alternatively a method of identifying a mutation which alters the activity of the transcription regulatory region the method comprising providing a DNA construct having a sequence encoding a reporter molecule operably linked to a DNA fragment comprising said regulatory region, and introducing said construct into a cell and monitoring for the level of expression of said reporter molecule. When the method is used to identify a variant which alters the activity of the transcription regulatory control region, the method may include the further step of comparing the levels of expression of a wild type and a polymorphic regulatory region as described herein.

According to each of the aspects of the invention, the regulatory region includes a polymorphic variation, preferably in a recognition site for a transcription factor of said regulatory region, and preferably in an activator protein-3 motif (AP-3) and/or a basic transcription element (BTE). In a preferred embodiment the variant occurs at position -475 or -147 of the region flanking the sequence encoding CYP3A5, and which region is illustrated in Figure 7. Preferably, both the variants are present.

The methods of the present invention will be particularly valuable to establish, prior to treatment with a drug, whether the drug will be effectively metabolised by the patient.

The invention may be more clearly understood by the following example with reference to the accompanying drawings wherein

Fig. 1a: is an illustration of the relationship between midazolam metabolic ratio and genotype for the linked A₋₁₄₇G and T₋₄₇₅G mutations in the 5' flanking region of the CYP3A5 gene. Midazolam metabolic ratio = 1-OHM/4-OHM, wt = samples with the wild type sequence in the 5' flanking region as previously published (11), Het = samples heterozygous for the linked polymorphisms, A₋₁₄₇G and T₋₄₇₅G.

Fig. 1b: is an illustration of the relationship between CYP3A5 mRNA expression and the linked A₋₁₄₇G and T₋₄₇₅G mutations in the 5' flanking region of CYP3A5. Relative Ct

difference = difference in threshold cycle
between samples, as described in the methods
section wt = samples with the wild type
sequence in the 5' flanking region as
5 previously published (11) Het = samples
heterozygous for the linked polymorphisms,
A₋₁₄₇G and T₋₄₇₅G.

10 Fig. 2a: is a diagram of relative position of
primers, and of the recognition site for the
restriction enzyme *Tai* I, which is
introduced into the PCR product utilising
mismatched primer 3A5R1 when the wild-type
"A" nucleotide is present at position -147,
15 and is lost when the mutant "G" nucleotide
is present.

20 Fig. 2b: is a diagrammatical representation of
expected restriction fragments for each
possible genotype for the A₋₁₄₇G variant,
i.e. homozygous wild-type, heterozygous and
homozygous mutant.

25 Fig. 2c: is an illustration of a 1.5% agarose gel of
Tai I restriction digest of 3A5F2/3A5R1 PCR
product for detection of the A₋₁₄₇G variant.
Lane 1. 100 bp ladder. Lanes 2 & 7.
Reference undigested PCR products. Lane 3.
30 Sample homozygous for the wild-type "A"
nucleotide at position -147. Lanes 10, 11,
16. Samples heterozygous for the A₋₁₄₇G
variant.

35 Fig. 3a: is a diagram of relative position of

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primers, and of the recognition sites for the restriction enzyme *Alu* I. The forward recognition site is introduced into the PCR product utilising mismatched primer 3A5F2 when the wild-type "T" nucleotide is present at position -475, and is lost when the variant "G" nucleotide is present.

Fig. 3b: is a diagrammatical representation of expected restriction fragments for each possible genotype for the T₋₄₇₅G variant, i.e. homozygous wild-type, heterozygous and homozygous mutant.

Fig. 3c: is an illustration of a 12.5% polyacrylamide ExcelGel of *Alu* I restriction digest of the 3A5F2/3A5R1 PCR product for detection of the T₋₄₇₅G mutation. Lane 1.100 bp ladder. Lanes 2, 5, 6, 7 & 8. Samples homozygous for the wild-type "T" nucleotide at position -147. Lanes 3, 4, 9. Samples heterozygous for the T₋₄₇₅G mutation. Fragment X - additional digestion product resulting from re-amplification of original template by primers 3A51/3A52.

Fig. 4a: is a comparison of 1-OHM/4-OHM metabolic ratios between samples with the linked mutations (HET group) and those wild-type for the mutations at positions -147 and -475 (WT group). Mean and quartiles are shown for each group, as is overall mean for the combined groups (central line).

Fig. 4b: is a comparison of CYP3A5 expression (ln

transformed) between samples with the linked mutations (HET group) and those wild-type for the mutations at positions -147 and -475 (WT group). Mean and quartiles are shown for each group, as is overall mean for the combined groups (central line).

Fig. 5: is a Western blot analysis of CYP3A5 protein expression in liver samples. A Western blot of microsomes prepared from liver samples and probed with a CYP3A5 specific antibody. Liver samples containing the linked polymorphisms at -147 and -475 (wt group) are marked * (sizes indicated in kDa from Wide Range Colour Marker (signs)).

Fig. 6: is a list of oligonucleotide mismatch primers used in accordance with the invention, where the underlined nucleotide indicates the sequence mismatch.

Fig. 7: is an illustration of the nucleotide sequence of the 5' flanking region relative to the DNA sequence encoding CYP3A5.

Fig. 8: is an illustration of the results obtained from an Electrophoretic mobility shift assay (EMSA) of A₋₁₄₇G oligonucleotide. EMSA was carried out as described in materials and methods. Lane 1.: A-147G oligonucleotide without HeLa nuclear extract; lanes 2-8 : in the presence of HeLa nuclear extract; lanes 3 and 4 : in the presence of 50 - 100 fold molar excess of unlabeled A-147G oligonucleotide; lanes 5 and 6; in the

presence of 50 - 100 fold molar excess of unlabeled wild type oligonucleotide; lanes 7 and 8 : in the presence of 1 and 2 microlitres of anti-Spl antibody.

5

Fig. 9-9d: are illustrations of the results obtained from the 'find patterns' program of the GCG sequence analysis package.

10

Experimental Procedures

Liver microsome preparation

15 Human liver samples were obtained from kidney transplant donors, and flash-frozen immediately on removal. Human liver microsomes were prepared according to previously described protocols (21), and protein content was determined by the method of Lowry
20 as modified by Miller (22).

Midazolam hydroxylase assay

25 The rates of midazolam overall metabolism and of the formation of 1- and 4-OH-midazolam were determined as follows. Each incubation vessel contained an aliquot of the microsomal suspension (containing 1 mg of microsomal protein) in 1.15 % KCl - 0.01 M phosphate buffer pH 7.4; 10 μ l of a stock solution of 6 mM
30 midazolam dissolved in DMSO to reach a final midazolam concentration of 60 μ M; 50.0 μ l of a co-factor mixture containing 0.5 mg of glucose-6-phosphate, 0.5 mg of $MgCl_2 \cdot 6H_2O$, 0.5 units of glucose-6-phosphate dehydrogenase dissolved in 0.5 M Na-K-phosphate
35 buffer, pH 7.4 and a 1.15 % KCl - 0.01 M phosphate

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buffer pH 7.4 to bring the incubation volume to 0.9 ml. After a pre-incubation for 5 min at 37°C, the incubations were started by adding 100 µl of a solution of 1.25 mg/ml NADP to reach a final concentration of 0.125 mg/ml. Tubes were continuously shaken at 100 oscillations/min in an Heto shaking waterbath. Blank incubates with boiled microsomes were incubated under identical conditions as the control incubates. The incubations were stopped after 30 min by immersing the tubes in dry ice. Samples were stored at $\leq -18^{\circ}\text{C}$ until analysis. The incubation samples were analysed for unchanged midazolam and for its metabolites 1'- and 4-hydroxymidazolam by HPLC with UV-detection.

HPLC determination of midazolam metabolites

The 1-ml samples of midazolam were thawed and diluted with 1 ml DMSO. Samples were sonicated for 10 min, centrifuged and an aliquot of the supernatant was injected directly onto the HPLC-column. The HPLC apparatus consisted of a Waters 600 MS pump. The samples were injected automatically, using a WISP 717 plus automatic injector. Stainless steel columns (30 cm x 4.6 mm i.d.) Were packed with Kromasill 18 (5 µm) bound phase by a balanced density slurry procedure (Haskel DSTV 122-C pump, 10^7 Pa). UV-detection at 230 nm was performed using a Waters 996 Diode Array Detector. Elution at 1-ml/min started with a short gradient from 100% 0.1 M ammonium acetate, pH 7.0 (solvent system A) to 50% of solvent system A and 50% of solvent system B containing 1M ammonium acetate pH 7.0, methanol and acetonitrile (10/45/45), over a 1-min period, followed by a second gradient to 100% solvent system B in 15 min. This solvent composition

was held for 2 min before equilibration with the starting conditions. The identity of the metabolites of midazolam was confirmed using mass spectroscopy. The conversion of UV-peak areas into ng was performed by a Millennium 2020 CDS system on a calibration curve of midazolam. This calibration curve was made up after injection of known amounts of the drug (0, 1059, 2117, 3176 and 5028 ng) and linear (weighted by 1/x) regression analysis of the corresponding UV-peak areas. The equation of the calibration curve was $ng = 0.000333 \times \text{area}$ ($r^2 = 0.9997$, $n = 5$). The metabolic activity was expressed as pmol metabolite formed/min mg protein, and a metabolic ratio was determined for each sample according to the ratio of 10HM/4OHM in each sample.

Genomic DNA preparation

DNA was isolated from frozen liver samples using a QIAmp Tissue Kit (QIAGEN) in accordance with the manufacturer's instructions.

RNA preparation

RNA was isolated from the liver samples using a QIAGEN RNeasy Midi Kit (QIAGEN), according to manufacturers instructions. Twenty μg of RNA was treated with RNase-free DNase I (Boehringer Mannheim), for 30 min at 37°C in 20 mM Tris-HCl, pH 8.0, 100 mM MgCl_2 . Samples were phenol/chloroform extracted, precipitated and resuspended in 30 μl of TE buffer. Two and a half μg of the treated sample was reverse transcribed for 50 minutes at 42°C in 1 x first strand buffer, 0.01M DTT and 0.5M dNTPs using 0.5 μg of oligo(dt) random primers and 200 units SuperScript II Reverse

Transcriptase (GibcoBRL) for use on the ABI Prism 7700 Sequence Detection System (SDS).

Sequencing of the CYP3A5 5' flanking region

5 A 1343 bp 5' flanking region of CYP3A5 was PCR amplified from genomic DNA isolated from liver samples, using primers 3A51 (5'-GGAAGCAACCTACATGTCCATC) and 3A52 (5'-ATCGCCACTTGCTTCTTC) based on the published sequence of Jounaidi et al. (11). PCR conditions were 1 cycle of 95°C for 1 min, 30 cycles of 95°C for 1 min, 57°C for 30 sec, 72°C for 2.5 min, and 1 cycle of 72°C for 10 min. PCR products were purified using a QIAquick PCR Purification Kit (QIAGEN), sequencing primers were designed (Table 1), and used to directly sequence the PCR product on both sense and antisense strands by cycle sequencing using the ABI BigDye Terminator cycle sequencing kit (Perkin Elmer). Sequencing reactions were analysed on an ABI 377 automated sequencer. Contig sequences were aligned and compared using the Sequence Editor version 1.0.3 software packages (Perkin Elmer) and manually edited for identification of heterozygote positions.

25 () ***PCR detection assays for the A₁₁₄G and T₄₇₅G mutations***

All PCR assays were performed utilising a 1 in 100 dilution of the original 3A51/3A52 PCR product as template, under the following conditions: 1 cycle of 95°C for 1 min, 30 cycles of 95°C for 1 min, 55°C for 30 sec, 72°C for 1 min, and 1 final cycle of 72°C for 10 min. All products were sequenced to confirm the identity of the product as CYP3A5. Oligonucleotide mismatched primers utilised in the assays were: 3A5F1

(5'-GGGTCTGTCTGGCTGGCGC), 3A5F2 (5'-GGGGTCTGTCTGGCTGAGC), and 3A5R1 (5'-TTATGTGCTGGAGAGGACG), where positions of mismatches are underlined.

5

For the A₁₄₇G mutation, PCR was performed using primer pair 3A5F2 and 3A5R1. Twenty μ l of PCR product was digested for a minimum of 3 hours at 65°C using 15 units of *Tai* I, and the restriction fragments visualised by ethidium bromide staining after electrophoresis on a 1.5% agarose gel.

10

For the T₄₇₅G mutation, PCR was performed using primer pair 3A5F2 and 3A5R1 as described above. Twenty μ l of PCR product was digested with 15 units of *Alu* I for a minimum of 3 hours, and restriction fragments were separated by electrophoresis on a 12.5% ExcelGel on a Pharmacia Multiphor Electrophoresis system (Pharmacia). Fragments were visualised by silver staining in a Hoeffer Automatic Gel Stainer (Pharmacia).

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To detect the presence of mutations on the same chromosome, PCR was performed using primers 3A5F1 and 3A5R1. Twenty μ l of PCR product was digested for a minimum of 3 hours at 65°C using 15 units of *Mvn* I, and the resulting restriction fragments were visualised by ethidium bromide staining after electrophoresis on a 1.5% agarose gel.

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Relative quantification and comparison of CYP3A5 RNA

Relative levels of CYP3A5 mRNA were determined by real time PCR using the ABI 7700 SDS (Perkin Elmer).

Optimal primers and probes for the detection of CYP3A5

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were designed using the PrimerExpress program, and subsequently checked to ensure specificity for CYP3A5. Primers utilised for the quantification PCR were: forward - 5'-AAGTGGCGATGGACCTCATC-3'; reverse - 5'-GAGGAGCACCCAGGCTGACA-3'. The TaqMan probe was labelled with the 5' reporter dye 6-carboxy-flouresine (FAM), and had the sequence 5'-CAAATTTGGCGGTGGAAACCTGGC-3'. Optimal primer/probe ratios and concentrations were determined and the experiments run according to standard protocols for the ABI 7700 Standard Detection System. CYP3A5 mRNA expression for all samples was normalised against the expression of β -actin mRNA. The threshold cycle (Ct) is the PCR cycle number where the ABI 7700 begins to detect an increase in fluorescent signal associated with the linear amplification of PCR product. The Ct value is dependent on the initial amount of template copy. Quantities of CYP3A5 in each sample were determined by averaging the Ct from 3 separate PCR reactions of each sample. Relative differences in Ct between samples were calculated by subtracting the Ct of each sample from the highest Ct within the samples (lowest expression). Since the amount of PCR product doubles with every cycle in the linear range of a PCR the differences in Ct were converted into estimated differences of mRNA quantity between the samples by calculating $2^{\delta Ct}$, where δCt is the difference in cycle threshold between two samples.

Negative controls were performed on each run to ensure that no signals were due to DNA contamination. Control samples consisted of RNA samples which had been treated in exactly the same manner as for the quantitative PCR, but without the addition of the reverse transcriptase.

Statistical Analysis

Statistical analysis was performed on the JMP
Statistical program version 3.2.2 (SAS Institute
5 Inc.). Metabolic ratio and CYP3A5 mRNA expression data
were checked to ensure that they conformed to a normal
distribution. CYP3A5 mRNA expression data did not
conform to a normal distribution and were ln-
transformed, after which the data was normally
10 distributed. Metabolic ratios and expression levels
were compared between groups using a t-Test.

Western Blot Analysis

15 Forty micrograms of microsomal protein prepared from
each liver were solubilised in an equal volume of
Laemmli sample buffer (Biorad) by four cycles of
freezing and boiling for 10 minutes. Samples were
loaded onto pre-cast 10% SDS-PAGE Ready Gels (Biorad)
20 and electrophoresed for 1 hour at 180 V. Separated
proteins were transferred onto Hybond-P membranes
(Amersham) using a Trans-blot SD apparatus (Biorad).
Membranes were blocked by an overnight incubation at
4°C in 1x PBS containing 5% (w:v) nonfat milk and 0.1%
25 (v:v) Tween. Membranes were incubated at ambient
temperature for 1 hour in a 1:3000 dilution of
specific human CYP3A5 antibody (Gentest) in 1x PBS,
2.5% nonfat milk, then rinsed four times in 1x PBS,
2.5% (w:v) nonfat milk, 0.1% (v:v) Tween. Membranes
30 were incubated at ambient temperature for 1 hour in a
1:5000 dilution of Anti-Rabbit IgG peroxidase
conjugate (Sigma) in 1x PBS, 2.5% (w:v) nonfat milk,
and rinsed as previously. The membranes were
developed using the ECL Plus Western Blotting
35 Detection System (Amersham) according to

manufacturer's instructions, and visualised by autoradiography using Kodak X-Omat film (sigma).

Example 1

5

Midazolam phenotyping

A panel of 39 liver samples was phenotyped for CYP3A5 activity, using the metabolism of midazolam to its 1-OH metabolite as a marker of activity. Human liver microsomal samples containing CYP3A5 in addition to CYP3A4 exhibit a significantly greater ratio of 1-OHM to 4-OHM compared with samples containing only CYP3A4. 1-OHM/4-OHM ratios between 5 and 9 were observed for microsomes containing both CYP3A4 and CYP3A5. Samples containing only CYP3A4 showed 1-OHM/4-OHM ratios < 4 (15). Analysis of the CYP3A5 phenotypes in our data set showed a clear bimodal distribution, with 6 samples (15%) having metabolic ratios greater than 5, and the remaining samples having metabolic ratios lying between 1.5 and 3.5 (see Fig. 1a). Of the 39 liver samples from which microsomes were prepared for metabolic analysis, sufficient tissue was available for full DNA and RNA analysis for 26, which included 6 samples lying in the higher metabolic ratio range. In addition to these 26 samples microsomes for protein analysis were available for a further 3 samples, all of which had metabolic ratios of <4.

Analysis of CYP3A5 gene 5' flanking region

The 5' flanking region of CYP3A5 was PCR-amplified from genomic DNA of all 26 samples and sequenced in full, as shown in Figure 7. Alignment showed that the region was well conserved. Only a small number of

inter-individual variations were identified in addition to a few variations from the published sequence (Table 2.). All variants detected were heterozygous, and all samples heterozygous for the more frequent A₋₁₄₇G mutation were also heterozygous for the T₋₄₇₅G mutation, suggesting that the two mutations were linked. These two mutations fall within two separate putative regulatory elements, a basic transcription element (BTE: A₋₁₄₇G) and an activator protein-3 motif (AP-3: T₋₄₇₅G). None of the remaining variants fell within putative regulatory domains.

PCR assays were developed to confirm the presence of the A₋₁₄₇G and T₋₄₇₅ mutations individually, and to ascertain if the two mutations were on the same, or on separate chromosomes. The PCR assay for the A₋₁₄₇G mutation was based on the creation of a recognition site for the restriction enzyme *Tai* I by utilising an oligonucleotide mismatch primer (3A5R1). This primer introduces a *Tai* I recognition site only when the wild-type "A" nucleotide is present at position -147. Digestion of the 369bp product with *Tai* I yields fragments of 349 and 20bp for the wild-type sequence, whilst the product remains undigested if the mutant "G" nucleotide is present (Fig. 2). Similarly, for the detection of the T₋₄₇₅G mutation a second oligonucleotide mismatch primer was used (3A5F2). This primer introduces a recognition site for the restriction enzyme *Alu* I when the wild-type T nucleotide is present at position -475, digesting the product to yield fragments of 318, 33 and 18 bp. This site is lost when the mutant G nucleotide is present, yielding digestion products of 336 and 33 bp (Fig. 3).

- 25 -

To determine if the mutations were present on the same chromosome a PCR assay was developed utilising two oligonucleotide mismatch primers (3A5F1 and 3A5R1), both primers introducing recognition sites for the restriction enzyme *Mvn* I when the mutant nucleotides are present at positions -147 and -475. If the mutations are present on the different chromosomes then the original 369 bp product is digested to yield products of 349/350 bp and 20/19 bp (inseparable by gel electrophoresis), whilst if present on the same chromosome the fragment is digested to yield products of 330 and 20/19 bp (data not shown). In addition to confirming the individual genotypes of the samples as determined by sequencing the two mutations were, in all cases, linked on the chromosome (data not shown).

Relationship between CYP3A5 allelic variants, CYP3A5 mediated metabolism, CYP3A5 mRNA and protein expression

Samples were grouped according to genotype: "Wild-type" or "mutant" (containing the linked polymorphisms), and the 1-OHM/4-OHM metabolic ratios (mr) were compared between the groups (Fig. 4a). With the exception of one outlier (liver sample number, mr = 2.08), all individuals carrying the linked mutations had metabolic ratios > 5.0, whilst the wild type group all possessed metabolic ratios of < 3.5. The mean metabolic ratios for the mutant group were significantly higher than those from the wild-type group (6.0 ± 2.0 versus 2.7 ± 0.42 , mean \pm standard deviation; $p < 0.001$).

Quantitative PCR was used to ascertain if the mutations in the 5' flanking region were affecting

gene expression. Whilst mRNA levels showed greater variation than the metabolic data, a degree of bimodality was observed (Fig. 1b). The mutant group had CYP3A5 mRNA levels skewed towards the higher end of the expression range, showing significantly higher levels of CYP3A5 mRNA than the wild type group (mean $\ln Ct = 4.03$, standard deviation = 0.97, against mean $\ln Ct = 2.06$, standard deviation = 1.2, $p < 0.006$) ((Fig. 4b). In this case the outlier (presenting with the mutant genotype, but wild type metabolic ratio) also fell within the lower range of expression ($\ln Ct = 2.9$).

The level of CYP3A5 protein expression levels was determined for 29 liver samples by Western blot analysis using a CYP3A5 specific antibody. A single band of 52 kDa corresponding to CYP3A5 was clearly apparent in some samples. With the exception of the single outlier with the high expression genotype (mutant) and low metabolic ratio phenotype (wild-type), all samples which possessed the high expression genotype, a high metabolic ratio and high RNA expression level clearly show high levels of CYP3A5 expression when compared to those samples with the low expression genotype and phenotype (Fig. 5). The single outlier with the high expression genotype, but low expression phenotype showed levels of CYP3A5 expression similar to those in the low expression genotype group. Longer exposure of the Western blot indicated that a very low level of CYP3A5 expression was apparent in most samples (data not shown).

The 5' flanking sequences of CYP3A5 obtained in this study are virtually identical to those published by Jounaidi et al. (11), and show little inter-individual

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variation in sequence. Interestingly, Jounaidi et al. sequenced two human genomic clones, one of which contained the two linked mutations described in detail in this report. This would suggest that one clone was
5 derived from an individual in the low expression group, and one from an individual in the high expression/metabolism group.

Previous studies had suggested that CYP3A5 was
10 expressed in 10-30% of livers (7, 8, 9) whilst another study has stated that some expression is constitutive in all samples (10). The present study supports the findings that some CYP3A5 expression is constitutive, with some metabolic activity and mRNA being detected
15 in all livers studied, although CYP3A5 protein was not convincingly demonstrated in all samples using the procedures required. We detected enhanced RNA and protein expression in 23% of the samples for which tissue was available (6 out of 26), which is similar
20 to the fraction of liver showing expression in previous studies. This supports the finding of Boobis et al. (10) that some show low level expression is constitutive in all liver samples although this can only be detected using more sensitive detection
25 techniques (such as PCR, and not by Western or Northern blot analysis).

Whilst both polymorphisms detected lie within putative transcriptional regulatory elements, we suspect that
30 the variant within the BTE is more likely to be responsible for altered expression since it has been reported that a BTE flanking the TATA box accounts for the constitutive expression of CYP1A1, and a similar region has been found in several other CYP genes
35 including CYP2B1, CYP2B2, CYP2E1 (16) CYP3A4 (13) and

CYP3A7 (12). In the case of CYP3A4 gene this element has been shown to bind nuclear extracts (13) and a basic transcription element binding factor for CYP3A7 (12), pointing to a role of this region in the general control of cytochrome P450 expression. The exact mechanism of up-regulation of CYP3A5 expression in the allelic variant described here remains to be determined although the presence of one of the mutations within the BTE, and the relevance of this element for the expression of other P450s indicates a possible mechanistic link. Using methylation interference footprinting, it has been shown that all guanine residues within the BTE, and other guanine residues in the vicinity, interacted with the transcriptional factor Sp1 (19). Given that the mutation within the BTE (Sp1) described herein alters an adenine residue to a guanine residue, then this could facilitate binding of transcription factors to the variant form of the Sp1.

Although there is considerable overlap in the range of CYP3A5 mRNA levels seen in the homozygous and heterozygous group, the distribution of metabolic ratios is clearly bimodal, as is the amount of CYP3A5. We cannot exclude the presence of other polymorphisms that may affect the translation efficiency or protein stability of CYP3A5. But given the better correlation between DNA polymorphism and protein level and the notorious liability of RNA, the simpler explanation is that differential RNA degradation or yield (due to differences in sample handling) has blurred the distinction between high and low expressers. Whatever the explanation for the discrepancy at the mRNA level, it does not in any way diminish the predictive value of the DNA polymorphism described.

- 29 -

There is, however, one individual whose genotype (heterozygous mutant) is not predictive of his metabolic phenotype (low expression). The fact that CYP3A5 protein as well as mRNA levels were low in this outlier indicates that the explanation must be sought at the transcriptional level, e.g. in transcription factors controlling CYP3A5 expression.

An AUG element in the 5'- untranslated region of the BTEB gene has been shown to be, at least in part, responsible for cell specific translational control of BTEB (20). Mutations within this region were shown to affect BTEB translation. Therefore, whilst the outlier in our study has a high expression genotype for CYP3A5 expression, this individual may have a "poor" expression phenotype for BTEB. Additionally, it is possible that a mechanism similar to that responsible for inducing CYP1A1 expression may also affect CYP3A5 expression. In addition to the BTE, CYP1A1 expression is mediated by a xenobiotic responsive element (XRE). In this case inducers enhance expression by binding to a cytosolic receptor (Ah receptor) which is translocated into the nucleus (possibly in association with an accessory protein coded for at the Arnt gene), where it binds the XRE (17, 18). Although variations in these and other transcription factors could further modulate CYP3A5 expression, this does not detract from the fact that the polymorphism described here seems to be the major determinant of CYP3A5 expression, at least in liver.

Despite the relatively small number of samples available for analysis in the present study, strong associations have been found between the two linked polymorphisms on the one hand and both expression and

- 30 -

CYP3A5 mRNA, protein and activity levels in the liver on the other hand. The unravelling of a genetic mechanism for the polymorphic metabolism by CYP3A5 will have important consequences in the field of pharmacogenetics. The ability to predict metabolism by genotyping will greatly facilitate disease association studies and may also help to explain adverse reactions or poor response to therapeutics which are metabolised by this cytochrome P450 isoform. It will also help in delineating which factors affecting CYP3A5 activity are genetic and which are environmental; for both further work will be required to fully understand the complex variation in expression observed with this enzyme.

Putative promoter sequence analysis

Materials and Methods:

The sequence of the regulatory region of CYP3A5 was analysed with the 'findpatterns' program of the GCG sequence analysis package (GCG, Madison, Wisconsin). This program finds specific DNA sequence motifs, patterns, and transcription binding sites, whose sequences are stored in the program, and are present in the sequence of interest. In the present analysis, at most one single mismatch or error per pattern is allowed in the sequence of interest, to detect if the two reported variations alter any known motifs or transcription binding sites. Results are identified in Figures 9 to 9d.

The first, GCGTG to GCTTG variation

removes binding sites for MBF-I_CS, MRE_CS2, and CNBP-SRE.

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The second, CCACC to CCGCC variation

replaces binding sites for apoE-undefined-site-3,
ApoE_B1, APRT-CHO_US, and APRT-human_US

by GCF-consensus, APRT-mouse_US, GC-box_(1),

5 DSE_(1), Sp1_CS4, Sp1-hsp70_(1), hsp70.2, Sp1-IE-
3.3, Sp1-IE-4/5, IRE_(1), Sp1-TPI_(4)

does not affect the Yi-consensus pattern

10 Both mutations affect transcription factor binding
sites.

Electrophoretic mobility shift assay (EMSA)

15 An EMSA was carried out using the Sp1 NUSHIFT Kit from
Geneka Biotechnology Inc. (Montreal, Canada) according
to the manufactures instructions. Briefly, a 31-mer
double-stranded oligonucleotide corresponding to the
CYP3A5 5'-untranslated region containing the A₋₁₄₇G
polymorphism (5'-GGC AGC TGC AGC CCC GCC TCC TTC TCC
20 AGC A-3') was end-labeled with ³²-P using T4
polynucleotide kinase. 50,000 cpm (0.5 ng)
oligonucleotide was incubated with 2 µg HeLa nuclear
extract for 30 min at 16°C. Unlabeled mutant or
wildtype (5-'GGC AGC TGC AGC CCC ACC TCC TTC TCC AGC
25 A-3') oligo nucleotide was added in 50-fold or 100-
fold excess as indicated. 1 or 2 µl anti-Sp1 rabbit
polyclonal antibody was pre-incubated with the nuclear
extract at 4°C for 30 min as indicated. Nuclear
30 extract, anti-Sp1 antibody and binding buffers were
from Geneka Biotechnology Inc. Samples were separated
on a 5% polyacrylamide (39:1) gel, in TGE buffer (25
mM Tris, 190 mM glycine, 1 mM EDTA, pH 8.3). The dried
gel was exposed to X-ray film.

35

RESULTS

Analysis of the 5'-untranslated region of the CYP3A5 gene indicated that the A₋₁₄₇G polymorphism might create a binding site for the transcription factor Sp1. An electrophoretic mobility shift assay (EMSA) was carried out to test this hypothesis. An oligo nucleotide containing the A₋₁₄₇G polymorphism was used to assay for binding factors present in HeLa nuclear extracts. A band shift was observed (Figure 8, lane 2) which was competed away with 50- and 100-fold excess respectively of unlabeled oligo nucleotide (Figure 8, lanes 3 and 4), but not with wildtype oligo nucleotide (Figure 8, lanes 5 and 6). This clearly indicates the presence of a protein factor in HeLa nuclear extracts capable of binding to the A₋₁₄₇G polymorphism region, but not to the wildtype region. Incubations in the presence of an antibody specific for the transcription factor Sp1 resulted in supershifting of the A₋₁₄₇G polymorphism oligo nucleotide (Figure 8, lanes 7 and 8), indicating that Sp1 is binding to the A₋₁₄₇G polymorphism site.

This change in binding affinity of transcription factor Sp1 to the 5'-untranslated region of the CYP3A5 gene might account for the increase in transcription from the A₋₁₄₇G polymorphic promoter and in turn, might contribute to the increase in metabolic rates correlated with the A₋₁₄₇G polymorphisms.

Genotyping of the cytochrome expression

A group of 300 healthy Caucasian volunteers was genotyped for variations T₋₁₇₃>G and A₋₁₄₇>G of the cytochrome P450 3A5 gene.

Test rationale

The first objective concerned allele/genotype frequencies.

5 Because the initial study included only 30 to 35 different individuals, allele/genotype frequencies could not be determined. Genotyping a group of 300 subjects should permit determination of these frequencies and to check whether they are in agreement
10 with the Hardy-Weinberg equilibrium.

The second objective concerned the linkage of the two variations. In the initial study, all samples with the gene variations $T_{-475} > G$ and $A_{-147} > G$ (only 6 in total) were linked. To verify the suggested linkage, both of
15 these CYP 3A5 polymorphisms were genotyped on a larger population.

Materials and methods

20 In order to minimize genotyping errors, genomic DNA samples from 300 healthy Caucasian volunteers were genotyped in a microtiterplate based format, which ensured a blind and completely independent duplicate analysis of each individual sample.

25 A 1343 bp 5' flanking region of CYP3A5 was PCR-amplified from genomic DNA using primers 3A51/3A52. PCR assays for both variations were performed using a 1/100 dilution of the original 3A51/3A52 PCR product as template. Mismatch primers 3A5F2 and 3A5R1 were
30 utilised for both assays. For the $A_{-147} > G$ mutation the PCR product was digested with restriction enzyme *Tai* I, and for the $T_{-475} > G$ mutation the PCR product was digested with restriction enzyme *Alu* I. After digestion the restriction fragments were separated by
35 polyacrylamide gel electrophoresis and visualised by

silver staining. The genotypes were determined based on the DNA fragment patterns by two independent observers.

5 Results

1. Allele/genotype frequencies

In the population of 300 individuals, 53 heterozygous subjects (18%) were carrying one copy of each of the variations, 246 subjects (82%) were homogenous for
10 A₋₁₄₇ and T₋₄₇₅, and one individual (0.3%) was carrying variations G₋₁₄₇ and G₋₄₇₅ on both alleles (homozygous). These frequencies are in agreement with 3A5 expression found in previous studies (7,8,9)

The allele frequencies are in agreement with the
15 Hardy-Weinberg equilibrium (Table 3).

2. Linkage of variations T₋₄₇₅ > G and A₋₁₄₇ > G

In all individuals, respectively variations T₋₄₇₅ and A₋₁₄₇, and variations G₋₄₇₅ and G₋₁₄₇, were equally
20 represented in genotypes, indicating a strong linkage between both variations. Whether this linkage between both variations has some functional significance needs to be clarified further. As a consequence of the linkage, future genotyping will require only the
25 analysis of one of the variations, whether it is the functional variant or not.

30

35

References

1. Cholerton, S., Daley, A.K., Idle, J.R., The role
of individual human cytochrome P450 in drug metabolism
5 and clinical response. *Trend Pharmac. Sci.* **13**, 434-
439 (1992).
2. Shimada, T., Guengerich, F.P. Evidence for
cytochrome P-450NF, the nifedipine oxidase, being the
10 principal enzyme involved in the bioactivation of
aflatoxins in human liver. *J. Biol. Chem.* **86**, 462-465
(1989).
3. Kormori M. et al. Fetus specific expression of a
15 form of cytochrome P-450 in human livers. *Biochemistry*
29, 4430-4433 (1990).
4. Hoyo-Vadillo, C. et al. Pharmacokinetics of
nifedipine slow release tablets in Mexican patients:
20 further evidence for an oxidative polymorphism. *J.*
Clin. Pharmac. **29**, 816-820 (1989).
5. Renwick, A.G., Robertson, D.R.C., Macklin, B.,
Challenor, V., Waller, D.G., George, C.F. The
25 pharmacokinetics of oral nifedipine - a population
study. *Br. J. Clin. Pharmacol.* **25**, 701-708 (1988).
6. Schellens, J.H.M., Soons, P.A., Breimer, D.D.
Lack of nifedipine plasma kinetics in a large
30 population of healthy subjects. *Biochem. Pharmacol.*
37, 2507-2510 (1988).
7. Aoyama, T., et al., Cytochrome P450 hPCN3 a novel
cytochrome P450IIIA gene product that is
35 differentially expressed in adult human liver. *J.Biol.*

Chem. **264**, 10388-10395 (1989).

8. Wrighton, S.A. et al. Studies in the expression and metabolic capabilities of human liver cytochrome P450IIIA5 (HLp3). *Mol. Pharmacol.* **38**, 207-213 (1990).
9. Schuetz, J., Beach, P., Guzelian, P.S. Selective expression of cytochrome P450 CYP3A mRNAs in embryonic and adult human liver. *Pharmacogenetics*, **4**, 11-20 (1994).
10. Boobis A.R., Edwards, R.J., Adams, D.A., Davies, D.S. Dissecting the function of cytochrome P450. *Br. J. Clin. Pharmacol.* **42**: 81-89 (1996).
11. Jounaidi, Y., Guzelina, P.S., Maurel, P., Vilarem, M.J. Sequence of the 5'-flanking region of CYP3A5 comparative analysis with CYP3A4 and CYP3A7. *Biochem. Biophys. Res. Commun.* **3**, 1741-1747 (1994).
12. Itoh, S., et al. Genomic organisation of human fetal specific P-450IIA7 (cytochrome P-450HFLa)-related gene(s) and interaction of transcriptional regulatory factor with its DNA element in the 5' flanking region. *Biochemica et Biophysica Acta.* **1130**, 133-138 (1992).
13. Hashimoto, H., et al. Gene structure of CYP3A4, an adult specific form of cytochrome P450 in human livers and its transcriptional regulation. *Eur. J. Biochem.* **218**, 585-595 (1993).
14. Barwick J.L., Quattrochi, L.C., Mills, A.S., Potenza, C., Tukey, Guzelian, P.S. Trans-species gene transfer for analysis of glucocorticoid-inducible

transcriptional activation of transiently expressed human CYP3A4 and rabbit CYP3A6 in primary cultures of adult rat and rabbit hepatocytes. *Molecular Pharmacology*, **50**, 10-16 (1996).

- 5
15. Gorski, J.C., Hall, S.D., Jones, D.R.,
VandenBranden, M., Wrighton, S.A. Regioselective
biotransformation of midazolam by members of the human
cytochrome P450 (CYP3A) subfamily. *Biochem. Pharmacol.*
10 **9**, 1643-1653 (1994).
16. Fujii-Kuriyama, Y., Imataka, H., Sogawa, K.,
Yasumoto, K.I., Kikuchi, Y. Regulation of CYP1A1
expression. *FASEB J.* **6**, 706-710 (1992).
- 15
17. Nebert, D.W. The Ah locus: genetic differences in
toxicity, Cancer, mutation and birth defects. *Critical
Reviews in Toxicology*, **20**, 153-174 (1989).
- 20
18. Hoffman, E.C., et al. Cloning of a factor
required for activity of the Ah (dioxin) receptor.
Science, **252**, 954-958 (1991).
- 25
19. Sogawa, K., Kikuchi, Y., Imataka, H., Fujii-
Kuriyama Y. Comparison of DNA-binding properties
between BTEB and Sp 1. *J. Biochem*, **114**, 605-9 (1993).
- 30
20. Imataka, H., Nakayama, K., Yasumoto, K., Mizuno,
A., Fujii-Kuriyama, Y., Hayami M. Cell specific
translational control of transcription factor BTEB
expression. The role of an upstream AUG in the 5'
untranslated region. *J. Biol. Chem.* **269**, 20668-73
(1994).
- 35
21. Lavrijsen K., Van Houdt, J., Thijs, D.,

- 5 Meuldermans, W., Heykants, J. Induction potential of antifungals containing an imidazole or triazole moiety. Miconazole and ketoconazole, but not itraconazole are able to induce hepatic drug metabolising enzymes of male rats at high doses. *Biochem. Pharmacol.* **35**, 1867-78 (1986).
- 10 22. Miller, G.L. Protein determination for large numbers of samples. *Anal. Chem.* **31**, 964 (1959).
23. Guengerich, F.P. Characterisation of human cytochrome P450 enzymes. *FASEB* **6**, 745-748 (1992).
- 15 24. Watkins, P.B. Drug metabolism by cytochromes P450 in the liver and small bowel. *Gastroenterology Clinics of North America*, **21**, 511-526 (1992).
- 20 25. Haehner, B.D. et al. Bimodal distribution of renal cytochrome P450 3A activity in humans. *Mol. Pharmacol.*, **50**, 52-59 (1996).

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30

35

Table 1. Primers used for sequencing 5' flanking region of CYP3A5 from PCR product 3A51/3A52 (see text).

Primer	Orientation#	Position*	Sequence (5'-3')
3A51	F	-1237- -1217	GGAAGCAACCTACATGTCCATC
3A5p01	F	-978- -963	AGTACAGGGAGCACAG
3A5p08	R	-917- -932	CACCTATTCATTCTG
3A5p02	F	-698- -684	TGCTATCACCACAGAC
3A5p07	R	-689- 704	GGTGATAGCAATAGAC
3A5p03	F	-364- -349	AGGATGTGTAGGAGTC
3A5p06	R	-417- -434	CCTCACACAGATGTAACC
3A5p04	F	-176- -161	TAAGAACTCAGGTTCC
3A5p05	R	-178- -194	CAGAAACTGAAGTGGAG
3A52	R	+105- +87	ATCGCCACTTGCCTTCTTC

F = 5' to 3', R = 3' to 5'

* Primer locations are based on CYP3A5 sequence data of Jounaidi et al (11)

Table 2.

Position	Variant Sequence	Percentage
-475	T-K (T or G) heterozygote	30.6% (11/36)
-147	A-R (A or G) heterozygote	30.6% (11/36)

TABLE 3.

Hardy Weinberg Equilibrium test

Test: CYP3A5 -45 A>G

Population: CON-JRF-1

		Observed values		Expected values	
		N	freq	N	freq
20	genotype AA	246	0.820	247.5	0.825
	genotype AG	53	0.177	50.0	0.167
	genotype GG	1	0.003	2.5	0.008
	total	300	1	300	1

1.112 = Chi-square (Pearson)

0.292 = p-value

1 = d.f.

	N	freq
Allele A	545	0.908
Allele G	55	0.092

CLAIMS

1. A method of identifying subjects having a high or low drug metabolising phenotype associated with cytochrome CYP3A5 expression, which method
5 comprises the steps of:

screening genomic DNA from said subject for the presence or absence of one or more polymorphic variants in a transcription regulatory region of the
10 sequence encoding CYP3A5 characteristic of a high drug metabolising phenotype.

2. A method of screening human subjects for suitability for treatment with a drug metabolised by CYP3A5 comprising screening for the presence or
15 absence of one or more polymorphic variants in a transcription regulatory region of the sequence encoding CYP3A5 characteristic of a high drug metabolising phenotype.

3. A method according to claim 1 or 2 comprising screening for said one or more variants in a recognition site for a transcription factor of said regulatory region.
20

4. A method according to any of claims 1 to 3 comprising screening for said one or more variants in an activator protein-3 motif (AP-3) and/or basic transcription element (BTE).
25

5. A method according to any of claims 1 to 4, comprising screening for said one or more variants at any one of positions -475 or -147 of the transcription regulatory region of the sequence encoding CYP3A5 the
30 sequence of which region is illustrated in Figure 7.
35

6. A method according to claim 5 comprising screening for both of said variants at position -475 or -147 of said transcriptional regulatory region of CYP3A5.

5

7. A method according to any of claims 1 to 5 wherein said DNA is amplified using oligonucleotide molecules which are capable of hybridising selectively to the wild type or variant sequences respectively such that generation of amplified DNA from said respective molecules will indicate whether said wild type or said variant is present.

10

8. A method of identifying one or more polymorphic variants in a transcription regulatory region of DNA encoding cytochrome CYP3A5 said method comprising the steps of:

15

1) subjecting the sample DNA to amplification using oligonucleotide molecules which are capable of selectively hybridising to the wild type and/or said one or more variant sequences, which molecules are such that they can introduce a restriction site in one of said amplified wild type or variant sequences, and

20

25

2) subjecting amplified DNA from step 1 to restriction with an enzyme which cleaves at said restriction site to provide a restriction digest indicative of the presence or absence of said mutation.

30

9. A method according to claim 8 wherein said molecule introduces a restriction site in a region corresponding to a recognition site for a transcription factor of said regulatory region.

35

10. A method according to claim 8 or 9 wherein said molecule introduces a restriction site in a region corresponding to an activator protein-3 motif (AP-3) and/or a basic transcription element (BTE).

5

11. A method according to claim 10 wherein said molecule is capable of introducing a restriction site only when the wild type A nucleotide is present at position -147 of the transcription regulatory region.

10

12. A method according to claim 11 wherein said restriction site is for the Tai I restriction enzyme.

13. A method according to claim 11 or 12 wherein said oligonucleotide molecule comprises the sequence designated 3A5R1 illustrated in Figure 6.

15

14. A method according to claim 10 wherein said molecule is capable of introducing a restriction site when the wild type T nucleotide is present at position -475 of the regulatory control region.

20

15. A method according to claim 14 wherein said restriction site is for the Alu I enzyme.

25

16. A method according to claim 14 or 15 wherein said molecule comprises the sequence designated 3A5F2 illustrated in Figure 6.

30

17. An oligonucleotide molecule of at least 10 contiguous nucleotides for use in amplification of a DNA sequence to detect a wild type or polymorphic variant in a transcription regulatory region of the sequence encoding cytochrome CYP3A5 said associated with a high or low drug metabolising phenotype

35

respectively, which molecule is capable of hybridising to a region incorporating either a polymorphic variant or wild type nucleotide in said region, such that amplification of said wild type and polymorphic variants will proceed from said molecule only when an oligonucleotide includes a sequence corresponding to either said wild type or polymorphic variant characteristic of a high drug metabolising phenotype.

18. A molecule according to claim 17 which is capable of hybridising to a recognition site for a transcription factor of said regulatory region.

19. A molecule according to claim 17 or 18 which is capable of hybridising to an activator protein-3 motif (AP-3) or a basic transcription element.

20. A molecule according to any of claims 17 to 19 which is capable of hybridising to a region comprising a polymorphic variant at any of positions -475 or -147 of the transcription regulatory region of the sequence encoding CYP3A5 illustrated in Figure 7.

21. A molecule according to any of claims 17 to 20 which comprises any of the sequences designated 3A5F1, 3A5F2 or 3A5R1 illustrated in Figure 6.

22. A kit for performing the method of any of claims 1 to 7 comprising an oligonucleotide molecule according to any of claims 17 to 21 and means for contacting said molecule and said transcription regulatory region of the sequence encoding CYP3A5.

23. A kit according to claim 22 further comprising a restriction enzyme capable of producing a

restriction digest for distinguishing between said variant or wild type sequences.

24. A kit according to claim 23 wherein said
5 enzyme comprises any of *Tai* I or *Alu* I.

25. A method of identifying toxic or mutagenic effects of a test compound, such as, a drug, toxin or procarcinogen metabolised by CYP3A5 the method
10 comprising contacting each of a cell having a high drug metabolising phenotype and a cell having a low metabolising phenotype associated with cytochrome CYP3A5 expression, with said test compound and
15 identifying the effects of said compound on each of said high or low drug metabolising phenotype cells or other cells sensitive to said compound.

26. A method of diagnosing susceptibility of an individual to a disease associated with environmental
20 toxins or procarcinogens metabolised by CYP3A5, which method comprises screening for the presence or absence of a polymorphic variant in a transcription regulatory region of the sequence encoding CYP3A5.

25 27. A method according to claim 26 comprising screening for said variant in a recognition site for a transcription factor of said regulatory region.

28. A method according to claim 26 or 27
30 comprising screening for said variant in an activator protein-3 motif (AP-3) and/or a basic transcription element (BTE) of said transcription regulatory region.

29. A method according to any of claims 26 to
35 28, comprising screening for said variant at any one

of position -475 or -147 of the transcription regulatory region of the sequence encoding CYP3A5 the sequence of which region is illustrated in Figure 7.

5 30. A method according to any of claims 26 to 29 comprising screening for both variants at position -475 or -147.

10 31. A method according to any of claims 26 to 30 comprising screening for the presence or absence of variants T₁₄₇G and A₁₄₇G in said transcriptional regulatory control region.

15 32. A method of providing a measure of activity of a transcription regulatory region of a DNA sequence encoding cytochrome CYP3A5 or of identifying a polymorphic variant which alters transcription of cytochrome CYP3A5, the method comprising providing a DNA construct having a sequence encoding a reporter molecule operably linked to a DNA fragment comprising said transcription regulatory region, and introducing said construct into a cell and monitoring for the level of expression of said reporter molecule.

25 33. A method of identifying transcription factors capable of hybridising to a DNA sequence from a transcription regulatory region adjacent to DNA encoding cytochrome CYP3A5, said method comprising contacting said DNA sequence including said transcription regulatory region with potential transcription factors and identifying any transcription factor complexed to said DNA sequence.

30 34. A method of identifying compounds acting on a transcription regulatory region of a DNA sequence

encoding CYP3A5, the method comprising transforming a cell with a DNA construct comprising the sequence of said regulatory region, and which regulatory region is operably linked to a sequence encoding a reporter molecule, contacting said cell with a test compound and identifying any altered expression of said reporter molecule.

35. A method of purifying transcription factors from a sample which are capable of binding to DNA from a transcription regulatory region of a sequence encoding cytochrome CYP3A5, the method comprising contacting a DNA sequence including said transcriptional regulatory region with a mixture of transcription factors and identifying any complexes of said transcription factors and said sequence.

36. A method according to any of claims 32 to 35 wherein said transcription regulatory region includes a mutation in a recognition site for a transcription factor of said regulatory region.

37. A method according to any of claims 32 to 36 wherein said mutation occurs in an activator protein-3 motif (AP-3) and/or a basic transcription element (BTE).

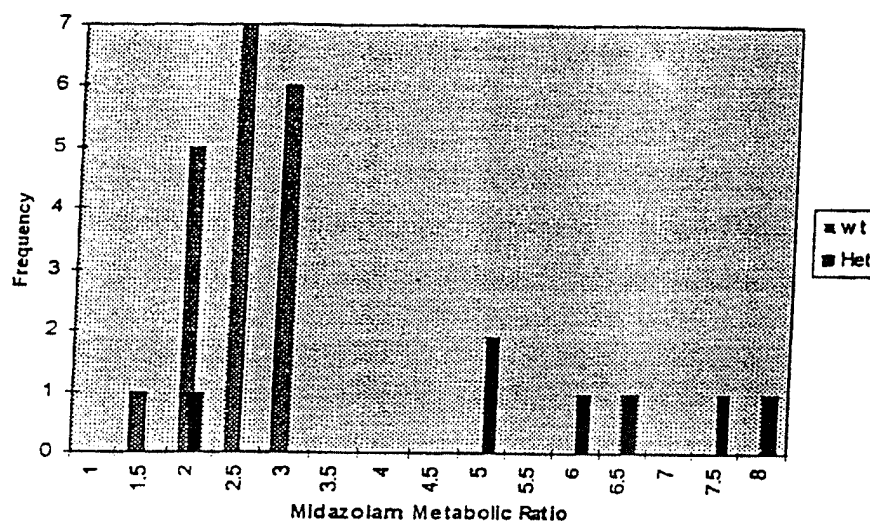
38. A method according to any of claims 36 or 37 wherein said mutation occurs at any one of positions -475 or -147 of the transcription regulatory region adjacent to the sequence encoding CYP3A5, the sequence of which region is illustrated in Figure 7.

39. A method according to any of claims 32 to 38 wherein the transcription regulatory region comprises

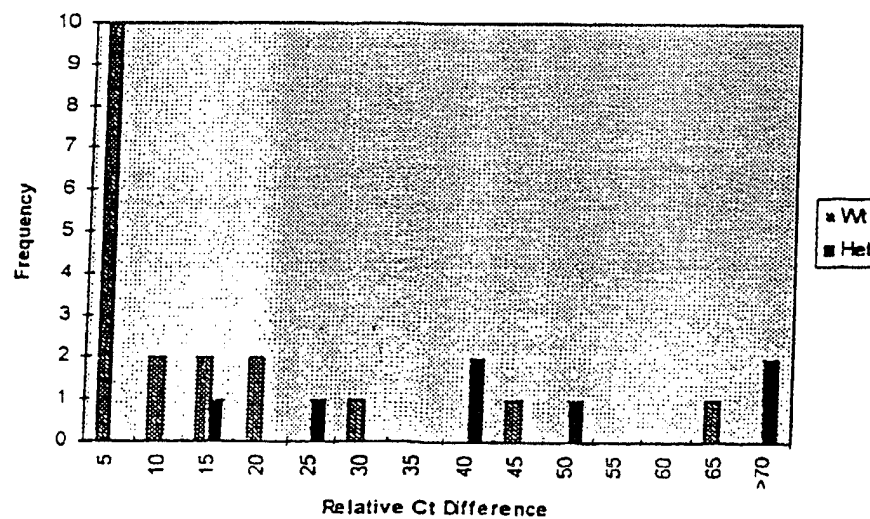
the mutations T₋₄₇₅G and A₋₁₄₇G.

FIG. 1a.

CYP3A5 Genotype/Phenotype Relationship

*FIG. 1b.*

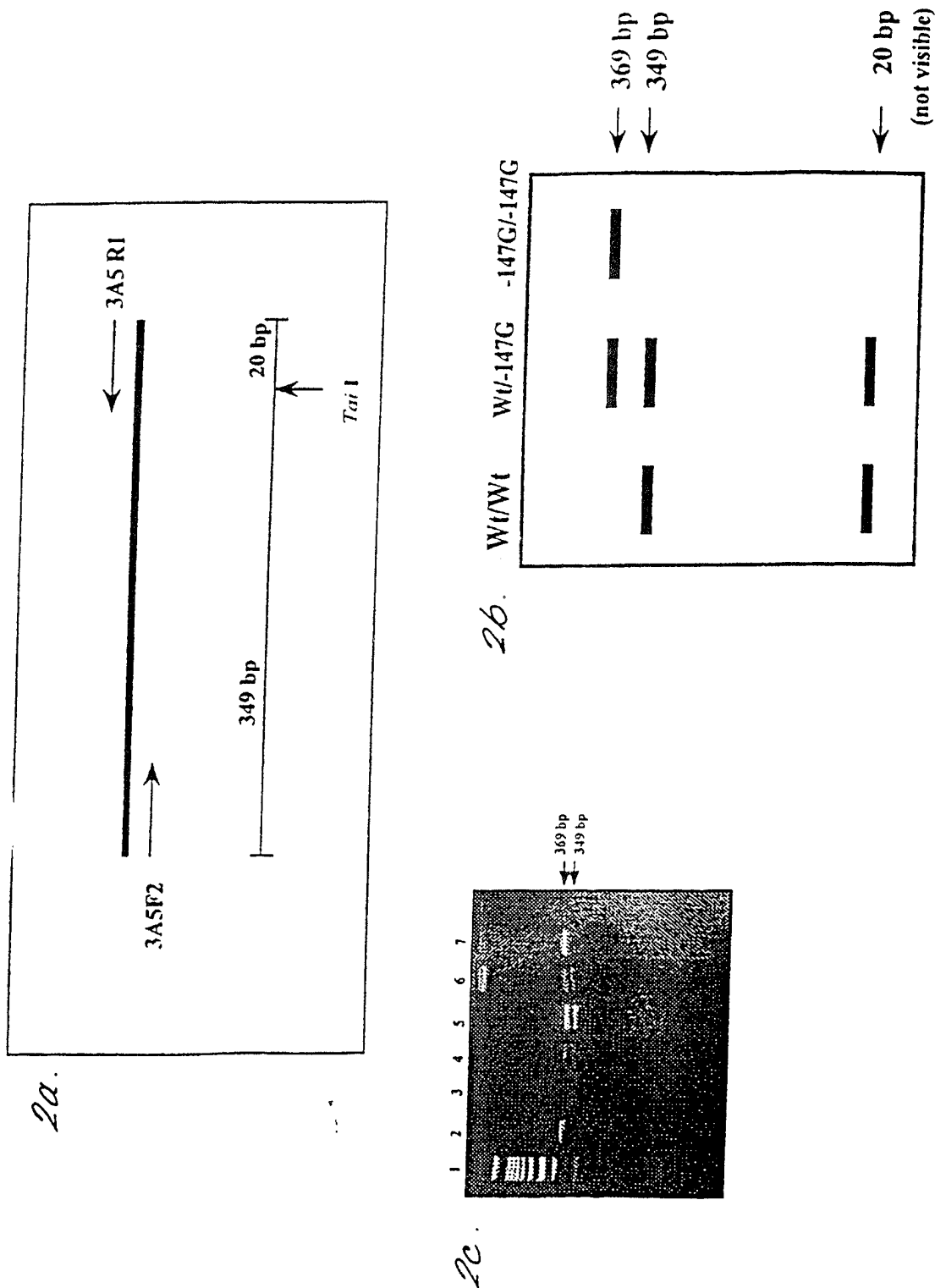
CYP3A5 mRNA Expression Related to Genotype



JC93 Rec'd PC 22 JUN 2001

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FIG. 2.

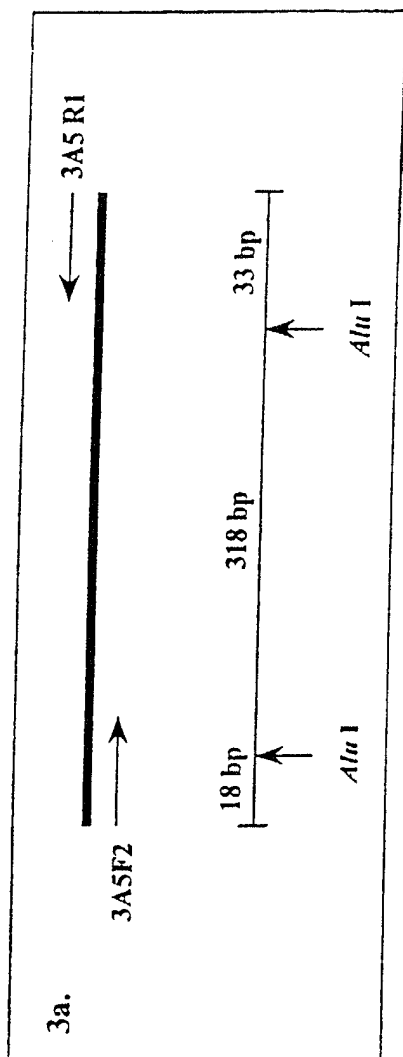


JC93 Rec'd PCT/PTC

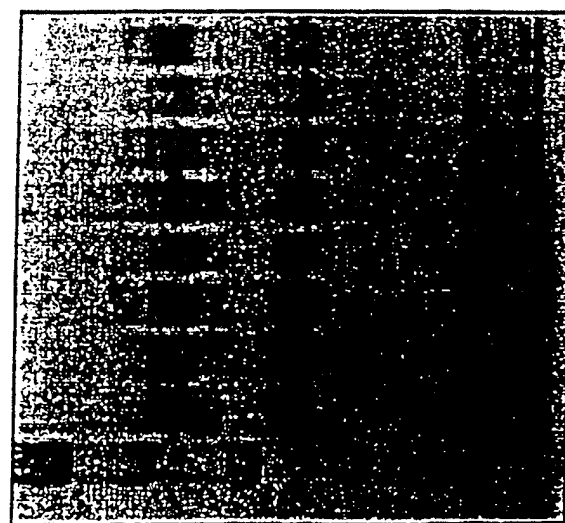
2 JUN 2001

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FIG. 3.



3c. 1 2 3 4 5 6 7 8 9



3b. Wt/Wt Wt/-475G -475G/-475G

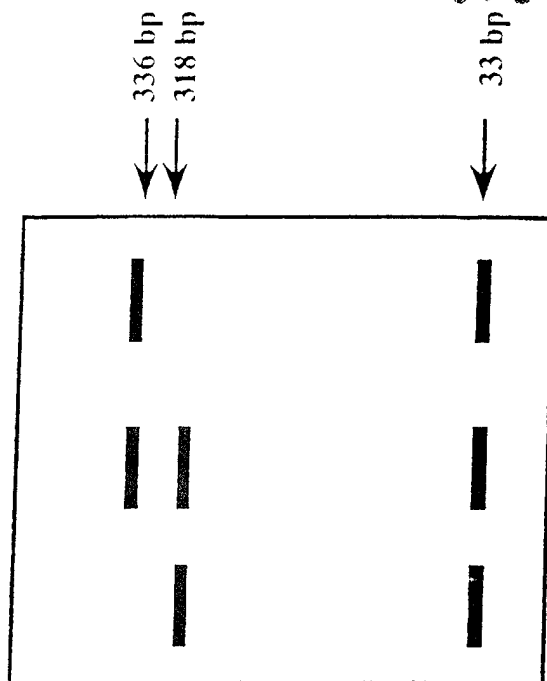
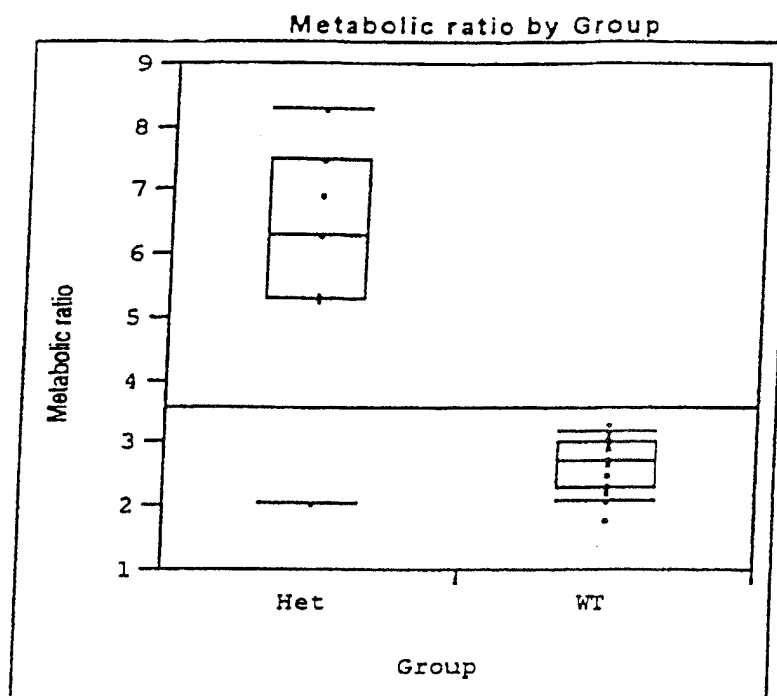
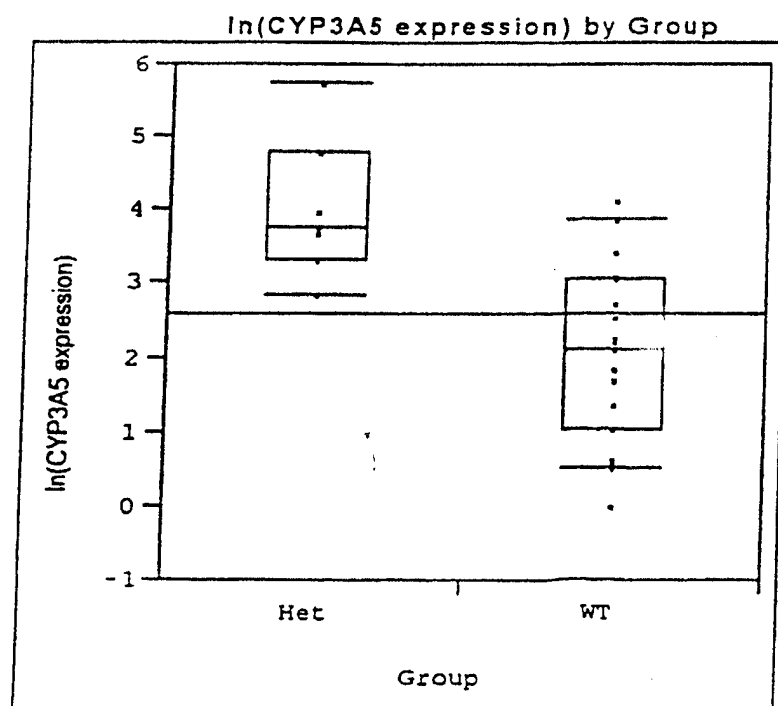
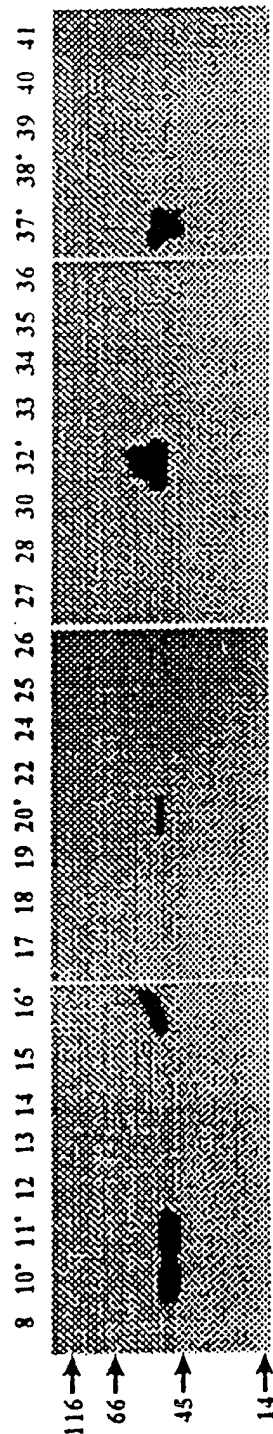


FIG. 4a.*FIG. 4b.*

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FIG. 5.



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FIG. 6.

3A5F1 5'-GGGTCTGTCTGGCTGCGC-3'

3A5F2 5'-GGGGTCTGTCTGGCTGAGC-3'

3A5R1 5'-TTTATGTGCTGGAGAAGGACG-3'

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FIG. 7.

-1343 GGAAGCAACC TACATGTCCA TCAACAGATG AATGGGTAAA GAGAGTACTT CACTTATGCA CAATGGAGTA
 -1273 CAATTCAGCC ATGAAAAAAG CATGAGATCC TGTCCCTTAT AATAACGTGG CTGGAACCTGC AGGTCATTAT
 -1203 GTTAGGTAAA ATAAGCCAGG CACACAAAAG CAGACATTGC ATGTTCTCAC TTATTGTGG GATCTACAAA
 -1133 TCAAAACAAT TGAGCTAATG TCTGGGTCTT AGTCAATTTT GTACCCTAAG TACAGGGAGC ACAGCCATTA
 -1063 GAATACATGA TGAATGCTTT AATACAGGAA TGAATAGGTG AGAGGCACAG GGTGGTTGGG TGTTCCTCTG
 -993 ATACATAGTA TCTTCCTTGA CACATTCAGT ACAACTCTCA ACAGGTAAGT CTCCTCATGT ATGTTACCTT
 -923 CTGAGGAATT AAGTGGCAGA ACATGCCCTT TATTATTTT CTTTGCAGAA CAAGACCAAT TGCATTAGTT
 -853 GGGAAACAGT GCTGGCTGCA TCTGAGCCCC AAGCAACCAT TAGTCTATTG CTATCACCAC AGACTCAGAG
 -783 GGGATGACAC ACAGGGGCCC AGCAATCTCA CCCAAGTCAA CTCCACCAAC ATTTCTGGTC ACCCACCATG
 -713 TGTACAGTAC CCTGCTAGGG TCCAGGGTCA TGAAAGTAAA TAATACCAGA CTGTGCCCTT GAGGAACCTCA
 -643 CCTCTGCTAA GGGAAACAGG CACAGAAACC CACAAGGGTG GTAGAGAGGA AATAGGACAA TAGGACTGTG
 -573 TGAGGGGGAT AGGAGGCACC CAGAGGAGGA AATGGTTACA TCTGTGTGAG GAGGTTGGTA AGGAAAGACT
 -503 TTAATAGAAG GGGTCTGTCT GGCTGGGCTT GCAAGGATGT GTAGGAGTCA TCTAGGGGGC ACAAGTACAC
 -433 TCCAGGCAGA GGGAAATTGCA TGGGTAAAGA TCTGCAGTTG TGGCTTGTGG GGATGGATTT CAAGTATTCT
 -363 GGAATGAAGA CAGCCATGGA AACAAGGGCA GGTGAGAGGA TATTTAAGAG GCTTCATGCC AATGGCTCCA
 -293 CTTCAGTTTC TGATAAGAAC TCAGGTTCCG TGGACTCCCT GATAAACTG ATTAAGTTGT TTATGATTCC
 -223 CCATAGAATA TGAACCTCAA GGAGGTAAGC AAAGGGGTGT GTGCGATTCT TTGCTACTGG CTGCAGCTGC

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FIG. 7 (CONTINUED)

-153 AGCCCCACCT CCTTCTCCAG CACATAAACA TTTCAGCAGC TTGACCTAAG ACTGCTGTGC AGGGCAGGGA

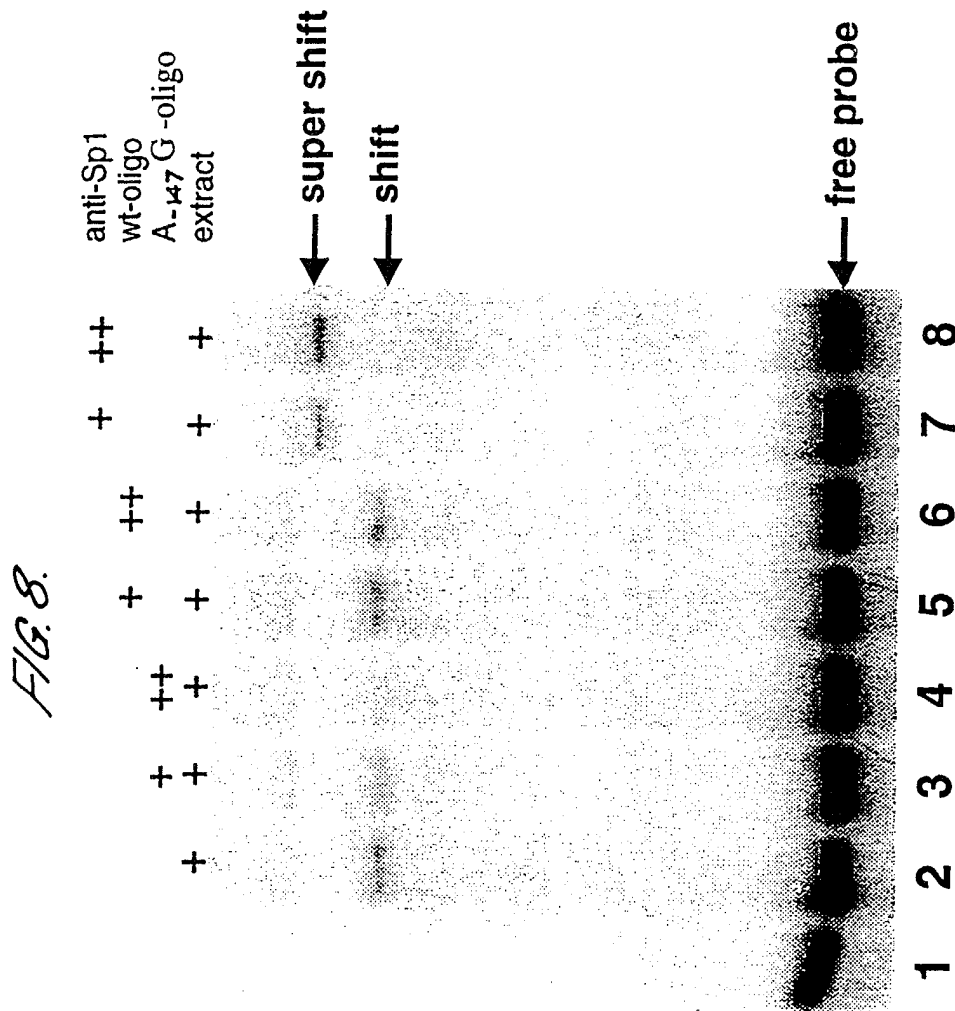
-83 TGCTCCAGGC AGACAGGCCCA GCAACAACA GCACACAGCT GAAAGTAAGA CTCAGAGGAG ACAGTTGAAG

-13 AAGGCAAGTG GCGATG

Variant Sequences in the 5' flanking region of CYP3A5

Position	Variant sequence	Percentage
-1317	G-K (T or G) heterozygote	2.8% (1/36)
-988	T-Y (C or T) heterozygote	8.3% (3/36)
-657	C-Y (C or T) heterozygote	8.3% (3/36)
-475	T-K (T or G) heterozygote	30.6% (11/36)
-264	G-R (G or A) heterozygote	2.8% (1/36)
-147	A-R (A or G) heterozygote	30.6% (11/36)

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CYP3A4, CYP3A5, CYP3A6/7

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FIG. 9 (CONTINUED 1).

CYP3A4, CYP3A5, CYP3A6/7

		*	440	*	460	*	480			
sites	:	-----		-----		-----		-		
HSCYPFLA_CYP3A6/7	:	-----		-----		-----		-		
HSRCYP3_CYP3A7	:	-----		-----		-----		-		
HSP4503A4_CYP3A4	:	GGGAGGGGG	ATTTTCG	AAAGCA	TGT	TAA	TAC	TTT	TTTCG	168
S74699_CYP3A5	:	AAATTCG	TAAAGT	TTGGGT	TTTGT	AAATTCG	TAAAGT	TTGGGT	TTTGT	332
S74700_CYP3A5	:	AAATTCG	TAAAGT	TTGGGT	TTTGT	AAATTCG	TAAAGT	TTGGGT	TTTGT	477
		*	500	*	520	*	540			
sites	:	-----		-----		-----		-		
HSCYPFLA_CYP3A6/7	:	-----		-----		-----		-		
HSRCYP3_CYP3A7	:	-----		-----		-----		-		
HSP4503A4_CYP3A4	:	AGGA	TTTCG	GGGCT	AGG	AGG	AGG	AGG	AGG	207
S74699_CYP3A5	:	AAATTCG	TAAAGT	TTGGGT	TTTGT	AAATTCG	TAAAGT	TTGGGT	TTTGT	391
S74700_CYP3A5	:	AAATTCG	TAAAGT	TTGGGT	TTTGT	AAATTCG	TAAAGT	TTGGGT	TTTGT	537
		*	560	*	580	*	600			
sites	:	-----		-----		-----		-		
HSCYPFLA_CYP3A6/7	:	-----		-----		-----		-		
HSRCYP3_CYP3A7	:	-----		-----		-----		-		
HSP4503A4_CYP3A4	:	AT	AAATTCG	TAAAGT	TTGGGT	TTTGT	AAATTCG	TAAAGT	TTGGGT	255
S74699_CYP3A5	:	GTTCGGT	TTTGT	AAATTCG	TAAAGT	TTGGGT	TTTGT	AAATTCG	TAAAGT	450
S74700_CYP3A5	:	GTTCGGT	TTTGT	AAATTCG	TAAAGT	TTGGGT	TTTGT	AAATTCG	TAAAGT	597
		*	620	*	640	*	660			
sites	:	-----		-----		-----		-		
HSCYPFLA_CYP3A6/7	:	-----		-----		-----		-		
HSRCYP3_CYP3A7	:	-----		-----		-----		-		
HSP4503A4_CYP3A4	:	TTTCG	GGGCT	AGG	AGG	AGG	AGG	AGG	AGG	303
S74699_CYP3A5	:	GGTAACT	TTTGT	AAATTCG	TAAAGT	TTGGGT	TTTGT	AAATTCG	TAAAGT	508
S74700_CYP3A5	:	G-TAACT	TTTGT	AAATTCG	TAAAGT	TTGGGT	TTTGT	AAATTCG	TAAAGT	656
		*	680	*	700	*	720			
sites	:	-----		-----		-----		-		
HSCYPFLA_CYP3A6/7	:	-----		-----		-----		-		
HSRCYP3_CYP3A7	:	-----		-----		-----		-		
HSP4503A4_CYP3A4	:	TGT	AAATTCG	TAAAGT	TTGGGT	TTTGT	AAATTCG	TAAAGT	TTGGGT	349
S74699_CYP3A5	:	TTTCG	GGGCT	AGG	AGG	AGG	AGG	AGG	AGG	568
S74700_CYP3A5	:	TTTCG	GGGCT	AGG	AGG	AGG	AGG	AGG	AGG	716
		*	740	*	760	*	780			
sites	:	-----		-----		-----		-		
HSCYPFLA_CYP3A6/7	:	-----		-----		-----		-		
HSRCYP3_CYP3A7	:	-----		-----		-----		-		
HSP4503A4_CYP3A4	:	TTTCG	GGGCT	AGG	AGG	AGG	AGG	AGG	AGG	391
S74699_CYP3A5	:	TTTCG	GGGCT	AGG	AGG	AGG	AGG	AGG	AGG	628
S74700_CYP3A5	:	TTTCG	GGGCT	AGG	AGG	AGG	AGG	AGG	AGG	776
		*	800	*	820	*	840			
sites	:	-----		-----		-----		-		
HSCYPFLA_CYP3A6/7	:	-----		-----		-----		-		
HSRCYP3_CYP3A7	:	-----		-----		-----		-		
HSP4503A4_CYP3A4	:	AGGCG	AGG	AGG	AGG	AGG	AGG	AGG	AGG	451
S74699_CYP3A5	:	AGGCG	AGG	AGG	AGG	AGG	AGG	AGG	AGG	688
S74700_CYP3A5	:	AGGCG	AGG	AGG	AGG	AGG	AGG	AGG	AGG	836

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FIG. 9 (CONTINUED 2)

CYP3A4, CYP3A5, CYP3A6/7

				*	860	*	880	*	900			
sites	:	-----								:	-	
HSCYPFLA_CYP3A6/7	:	-----								:	-	
HSRCYP3_CYP3A7	:	-----								:	-	
HSP4503A4_CYP3A4	:	GTA	AGTA	TTGGT	AGGGT	ATGG	AGGGT	ATGA	AGTA	TTGG	:	511
S74699_CYP3A5	:	GTA	AGTA	TTGGT	AGGGT	ATGA	AGGGT	ATGA	AGTA	TTGG	:	747
S74700_CYP3A5	:	GTA	AGTA	TTGGT	AGGGT	ATGA	AGGGT	ATGA	AGTA	TTGG	:	893

[illegible]

		*	980	*	1000	*	1020	
sites	:	-----						:
HSCYPFLA_CYP3A6/7	:							:
HSRCYP3_CYP3A7	:							:
HSP4503A4_CYP3A4	:							:
S74699_CYP3A5	:							:
S74700_CYP3A5	:							:

CGGGG	AAATGGG	TTC	TAAG	GGGGG	TGGG	CTGTG		GGGGG	GG	GGTGG	TTT	:	629
GGGGG	AAATGGG	TTC	TGTC	GGGGG	TGGG	GGG		GGGGG	GG	GGTGG	TTT	:	867
ATGGG	AAATGGG	TTC	TGTC	GGGGG	TGGG	GGG		GGGGG	GG	GGTGG	TTT	:	1010

```

*          1040          *          1060          *          1080
sites      : ----- : -
HSCYPFLA_CYP3A6/7 : ----- : -
HSRCYP3_CYP3A7    : ----- : -
HSP4503A4_CYP3A4  : TTGTTGTTGGGGGTTGGTTGGGATGCTTTAGGGGGGGTTCCTCTTGGGGTTG : 688
S74699_CYP3A5     : TTGTTGAGGGGTTGGTTGGGATGCTTTAGGGGGGGTTCCTTCGTTGGGTGG : 927
S74700_CYP3A5     : TTGTTGAGGGGTTGGTTGGGATGCTTTAGGGGGGGTTCCTTCGTTGGTGG : 1068

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* 1100 * 1120 * 1140
 sites : ----- : -
 HSCYPFLA_CYP3A6/7 : ----- : -
 HSRCPY3_CYP3A7 : ----- : -
 HSP4503A4_CYP3A4 : GAGGGATCTGTATGGAGTATATGGGGGATAGGATAGGAGGAGGGGATAG : 736
 S74699_CYP3A5 : GAGGGATCTGTATGGAGTATATGGGGGATAGGATAGGAGGAGGGGATAG : 987
 S74700_CYP3A5 : GAGGGATCTGTATGGAGTATATGGGGGATAGGATAGGAGGAGGGGATAG : 1128

	*	1160	*	1180	*	1200	
sites	:	-----					:
HSCYPFLA_CYP3A6/7	:	-----					:
HSRCYP3_CYP3A7	:	-----					:
HSP4503A4_CYP3A4	:	-----					:
S74699_CYP3A5	:	-----					: 796
S74700_CYP3A5	:	-----					: 1046
	:	-----					: 1187

[illegible]

FIG. 9 (CONTINUED 3)

CYP3A4, CYP3A5, CYP3A6/7

```

sites
HSCYPFLA_CYP3A6/7 :
HSRCYP3_CYP3A7 :
HSP4503A4_CYP3A4 :
S74699_CYP3A5 :
S74700_CYP3A5 :
1280 1300 1320
916 1165 1307

sites
HSCYPFLA_CYP3A6/7 :
HSRCYP3_CYP3A7 :
HSP4503A4_CYP3A4 :
S74699_CYP3A5 :
S74700_CYP3A5 :
1340 1360 1380
976 1224 1366

sites
HSCYPFLA_CYP3A6/7 :
HSRCYP3_CYP3A7 :
HSP4503A4_CYP3A4 :
S74699_CYP3A5 :
S74700_CYP3A5 :
1400 1420 1440
1036 1242 1383

sites
HSCYPFLA_CYP3A6/7 :
HSRCYP3_CYP3A7 :
HSP4503A4_CYP3A4 :
S74699_CYP3A5 :
S74700_CYP3A5 :
1460 1480 1500
1115 1096 1286 1427
gc c
ata

sites
HSCYPFLA_CYP3A6/7 :
HSRCYP3_CYP3A7 :
HSP4503A4_CYP3A4 :
S74699_CYP3A5 :
S74700_CYP3A5 :
1520 1540 1560
174 1156 1345 1486
gg cagg gctcca ca a agcccagcaaa a ca c

sites
HSCYPFLA_CYP3A6/7 :
HSRCYP3_CYP3A7 :
HSP4503A4_CYP3A4 :
S74699_CYP3A5 :
S74700_CYP3A5 :
1580 1600
70 94 228 1210 1399 1540
ac gctgaaa aagactcagaggaga ag t a aagg aaGT G gATG

```

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FIG. 9(a).

```

      A or G
      =
MEME repeated motif 9
=====
      MEME 'single' motif 9
=====
      Yi-consensus
      =====
      apoE-undefined-site-3
      =====
      ApoE_B1
      =====
      APRT-human_US
      =====
      APRT-CHO_US
      =====
1238  AGCTGCAGCCCCACCTCCTTCTCCAGC
      TCGACGTCGGGGTGGAGGAAGAGGTCG
      .....

```

(B) A

FIG. 9(b).

```

MEME repeated motif 2
=====
MEME repeated motif 2
=====
      MEME 'single' motif 9
=====
      Yi-consensus
      =====
      Sp1-TPI_(4)
      =====
      GCF-consensus
      =====
      DSE_(1)
      =====
      IRE_(1)
      =====
      Sp1_CS4
      =====
      GC-box_(1)
      =====
      Sp1-IE-4/5
      =====
      Sp1-IE-3.3
      =====
      E2A_CS    hsp70.2
      =====
      E2A_CSSp1-hsp70_(1)
      =====
      APRT-mouse_US
      =====
1379  AGCTGCAGCCCCGCTCCTTCTCCAGC
      TCGACGTCGGGGCGGAGGAAGAGGTCG
      .....

```

(B) G

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FIG. 9(c).

```

MEME repeated motif 9
=====
MEME repeated motif 9
=====
      MEME repeated motif 3
      =====
      MEME 'single' motif 6
      =====
      E-2.7_kb_(3)
      =
      E1A-F_CS
      =====
      GH1          MTVGRE_NRS
      =====
910  TCTGTCTGGCTGGGCTTGCAAGGATGTGTAG  (A) T
      AGACAGACCGACCCGAACGTTCTACACATC
.....

```

FIG. 9(d).

```

MEME repeated motif 9
=====
MEME repeated motif 9
=====
MEME repeated motif 9
=====
      MEME repeated motif 3
      =====
      MEME 'single' motif 6
      =====
      E-2.7_kb_(3)
      =
      MBF-I_CS
      =====
      E1A-F_CS
      =====
      CNBP-SREMTVGRE_NRS
      =====
      GH1          MRE_CS2
      =====
1052 TCTGTCTGGCTGGCGTGCAAGGATGTGTAG  (A) G
      AGACAGACCGACCGCACGTTCTACACATC
.....

```